Production of Mutagenic Metabolites by *Metarhizium anisopliae*

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NG-391 (**1**) and NG-393 (**2**), previously reported from undescribed *Fusarium* species as nerve-cell growth stimulants, were identified from fermentation extracts of the entomopathogenic fungus *Metarhizium anisopliae*. These compounds are 7-desmethyl analogues of fusarin C and (8*Z*)-fusarin C, mutagenic toxins from *Fusarium* species that contaminate corn. A mutant strain of *M. anisopliae* (KOB1-3) overproduces **1** and **2** by ca. 10-fold relative to the wild-type strain, ARSEF 2575, from which it was derived. Overproduction of these compounds in KOB1-3 imparts a yellow pigmentation to the culture medium of the fungus. These compounds were inactive at 100 μg/disk in antimicrobial disk diffusion assays. Compound **1** was inactive at 100 ppm in a mosquitocidal assay. However, like their fusarin analogues, **1** and **2** exhibited potent S9-dependent mutagenic activity in the *Salmonella* mutagenicity test. Discovery of these highly mutagenic mycotoxins in *M. anisopliae* suggests that screening for production of NG-391 and NG-393 in strains that are used as biocontrol agents would be a prudent course of action. The impact of these findings on the use of *M. anisopliae* as a biocontrol agent is currently unknown and requires further investigation.

KEYWORDS: NG-391; NG-393; fusarin C; *Metarhizium anisopliae*; mutagen

INTRODUCTION

Fungi in the genus *Metarhizium* cause fatal mycoses in a wide variety of important insect pest species and, consequently, occupy the forefront of efforts to develop entomopathogenic fungi as biological control agents. Recently, *Metarhizium anisopliae* strain F52 has been developed commercially in the United States for control of weevils (*1*) and other pests (*2*). In other countries, numerous laboratories have mounted intensive efforts to develop strains of *M. anisopliae* for inundative applications against agricultural pests (*3*). Most notably, Green Muscle, a product based on *M. anisopliae* var. *acridum*, was developed by the international LUBILOSA program for locust and grasshopper control in Africa (*3*).

For microbial strains already in use or under evaluation as biocontrol agents, it is especially important to identify all possible bioactive secondary metabolites from a regulatory standpoint, as well as to further understand how various products serve the producing organism (*4*). *Metarhizium* species produce a variety of secondary metabolites, preeminently the destruxins, cyclic depsipeptides possessing a broad range of toxic effects, including acute toxicity to insects (*5*−*7*). Metabolites reported to date from *Metarhizium* species include cytochalasins C and D (*8*), helvolic acid (*9*), swainsonine (*10*), 12-hydroxyovalicin (*11*), viridoxin (*12*), myroridins (*13*), and hydroxyfungerin (*14*).

While screening for destruxin production by mutants of a wild-type (WT) strain of *M. anisopliae* (ARSEF 2575) in which a nonribosomal peptide synthetase (NPS) gene (*NPS1*) was disrupted (*15*), we observed that mutant strain KOB1-3, which carries at least one additional uncharacterized mutation distinct from the disrupted *NPS1* gene, exhibited strong yellow color in liquid culture and produced prominent interference peaks in HPLC analyses for destruxins that were not evident in comparable analyses of WT and other mutants. We isolated the two major components of a complex pigment mixture and identified them as NG-391 (**1**) and NG-393 (**2**) (**Figure 1**), two polyene-substituted pyrrolidinones previously identified from two undescribed *Fusarium* species (*16*, *17*). Herein, we describe details of the isolation and identification of these compounds from *M. anisopliae*, present evidence of their overproduction by KOB1-3, and explore their mutagenic, antimicrobial, and insecticidal activity.

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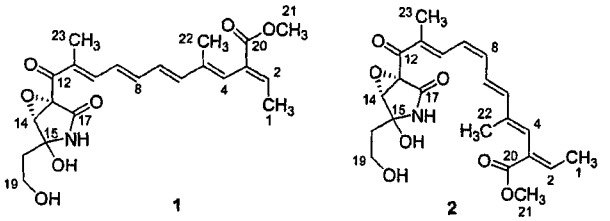

Figure 1. Structures of NG-391 (**1**) and NG-393 (**2**).

MATERIALS AND METHODS

Polarimetry. Optical rotations were measured at 25 °C in MeOH on a Perkin-Elmer 241 polarimeter using the sodium lamp (589 nm) with a 100 mm cell.

Analytical HPLC. HPLC retention times were determined on a 250 mm × 4.6 mm, 5 μm, 100 Å Fluophase column (Thermo Electron Corp., Bellefonte, PA) using a premixed isocratic mobile phase (9:16 MeCN/H_2O mixture, flow rate of 1 mL/min) with detection by UV absorption at 215 and 363 nm using a model 2487 dual-wavelength detector (Waters Corp., Milford, MA).

Mass Spectrometry. Low-resolution ESI mass spectra were acquired by infusion of methanolic solutions at 5 μL/min via a syringe pump (Harvard Apparatus, Holliston, MA) into a Micromass ZMD 4000 spectrometer (Waters Corp.). Positive ion mode spectra were recorded with capillary and cone voltages of 3.4 kV and 50 V, respectively. High-resolution ESI mass spectra were acquired on a Micromass QTOF Ultima spectrometer with capillary and cone voltages of 3.0 kV and 35 V, respectively.

NMR Spectroscopy. All NMR spectra were acquired at 600 MHz on a Varian Inova 600 spectrometer using an HCN inverse detection probe. HMQC, HSQC and HMBC, gCOSY, dqfCOSY, NOESY, and ROESY experiments were carried out using standard Varian pulse sequences. HMQC and HSQC experiments were optimized for a $^1J_{CH}$ of 150 Hz. HMBC experiments were optimized for $^nJ_{CH}$ values of 5.0 and 8.0 Hz. NOESY experiments employed a mixing time of 600 ms. ROESY experiments were carried out with a spin-lock time of 200 ms. Spectra acquired in $CDCl_3$ were referenced to the residual $CHCl_3$ singlet at δ 7.26 and the $^{13}CDCl_3$ resonance detected in HMQC spectra at δ_C 77.23. Spectra acquired in CD_3OD were referenced to the centers of the residual CHD_2OD quintuplet at δ 3.31 and the $^{13}CD_3OD$ signal at δ_C 49.15 detected in HMQC spectra.

Fungal Material and Culture Conditions. Derivation of *NPS1* knockout (KO) mutant strains KOB1-3 and KO8-18-1 and ectopic control transformant EctA-18 from WT *M. anisopliae* strain ARSEF 2575 was described previously (*15*). Cryogenically preserved mycelium was used to inoculate stock cultures maintained on plates of 1/4-strength Sabouraud's dextrose agar plus yeast extract (0.25%) which were in turn used to provide inoculum for liquid cultures grown in Czapek Dox broth (BD-Difco, Inc., Sparks, MD) with bactopeptone (0.5%) (CDB). For isolation of compounds, 1 L batches of CDB in 2 L Fernbach flasks were inoculated with plugs from 10-day-old solid cultures of KOB1-3 and were fermented with rotary shaking at 160 rpm and 24 ± 2 °C for 13 days; for quantitative HPLC analyses, 100 mL batches of CDB in 250 mL Erlenmeyer flasks were inoculated with 1 mL of a suspension in H_2O (10^6 spores/mL) and were grown with rotary shaking at 160 rpm and 24 ± 2 °C for 11 days. Cultures were harvested by filtering mycelium from broth through four layers of cheesecloth.

Extractions and Isolation. The culture filtrate from four 1 L batches of KOB1-3 was extracted with three 500 mL portions of CH_2Cl_2 per liter of broth. The organic layers were combined and dried over anhydrous Na_2SO_4, and the solvent was removed in vacuo to afford an oily brown residue (513 mg). This crude extract was redissolved in MeOH, applied to a 850 mm × 25 mm column of Sephadex LH-20, with a bead size of 25−100 μm (Sigma Chemical Co., St. Louis, MO), and chromatographed by elution with MeOH at a flow rate of 4.5 mL/min. Two-minute fractions were collected and recombined on the basis of ESI-MS analysis. Destruxins eluted in a band prior to two distinct yellow bands. Fractions from the later-eluting yellow band, which exhibited a predominant ESI-MS peak at *m/z* 440 corresponding to the pseudomolecular ion, $[M + Na]^+$, of **1** and **2**, were combined and concentrated in vacuo to afford 54 mg of a yellow oil. This was then dissolved in MeOH and further fractionated by repeated semipreparative HPLC using a 250 mm × 10 mm, 5 μm, 100 Å Fluophase RP column (Thermo Electron), eluting with a MeCN/H_2O mixture (2:3, v/v) at a flow rate of 4 mL/min, with detection by UV absorption at 215 nm, to afford fractions containing 60% pure **1** (9.9 mg) and ~85% pure **2** (5.4 mg). A final round of HPLC using a 250 mm × 10 mm, 5 μm, 100 Å RPC18 ODS3 Prodigy column (Phenomenex, Torrance, CA) eluting with a MeCN/H_2O mixture (2:3, v/v) afforded >90% pure **1** (3.3 mg). HPLC using the 250 mm × 10 mm Fluophase RP column with a 9:16 MeCN/H_2O mixture afforded >95% pure **2** (3.3 mg). All samples were handled under reduced light and with minimal warming to minimize UV- and heat-mediated transformations as observed with the fusarins (*18*).

Compound 1 (NG-391): yellow oil; $[\alpha]^{25}_D$ 76.1° (*c* = 2.7 mg/mL, MeOH); HPLC t_R = 9.5 min; HRESI-MS *m/z* $[M + H]^+$ 418.1860 (calcd for $C_{22}H_{28}NO_7$, 418.1866). For ^1H NMR, ^{13}C NMR, HMBC, COSY, and NOESY data, see **Table 1**, **Figure 2**, and the Supporting Information.

Compound 2 (NG-393): yellow oil; $[\alpha]^{25}_D$ 181.6° (*c* = 2.7 mg/mL, MeOH); HPLC t_R = 8.9 min; HRESI-MS *m/z* $[M + H]^+$ 418.1859 (calcd for $C_{22}H_{28}NO_7$, 418.1866). For ^1H NMR, ^{13}C NMR, HMBC, COSY, and NOESY data, see **Table 1**, **Figure 2**, and the Supporting Information.

Quantitative HPLC Analysis. Fermentation filtrates from liquid cultures of WT and mutant strains of ARSEF 2575 were extracted in CH_2Cl_2 as described above. Mycelial mats were blended in EtOH and stirred for 24 h at 25 °C. The ethanolic extract was then filtered and concentrated to dryness in vacuo, and the residue was partitioned between CH_2Cl_2 and water. The organic layer was dried over anhydrous sodium sulfate and concentrated in vacuo. The mycelial mat was oven-dried to obtain a dry weight. All extracts were dissolved in 1 mL of MeOH. The sample was then filtered and diluted 10-fold, and a 10 μL aliquot of the dilution (0.1 mL of broth equivalent or 0.1% of the sample) was analyzed by HPLC using a 250 mm × 4.6 mm, 5 μm, 100 Å Fluophase RP column (Thermo Electron Corp.) eluting with a MeCN/H_2O mixture (2:3, v/v) at a flow rate of 1 mL/min with detection by UV absorption at 363 nm. Quantitative estimates of **1** and **2** were made from standard curves for each compound constructed from six points bracketing the range of peak areas observed in extracts. Using a signal-to-noise ratio (S:N) of 5, this method provided limits of detection (LOD) of 1.8 and 0.9 ng for **1** and **2**, respectively. Limits of quantification (LOQ) were established at twice the LODs (S:N ratio = 10). Estimates for both broth and mycelial extracts were calculated as micrograms per gram of mycelial dry weight.

Microbiological Assays. Effects of fungal fermentation extracts, chromatographic fractions, and purified compounds were tested against bacterial target strains (*Escherichia coli* and *Bacillus cereus*) grown on nutrient agar plates (9 cm) and against fungal target strains (*Colletotrichum acutatum* and *Botrytis cinerea*) grown on potato dextrose agar plates. Plates spread with 0.6 mL aliquots of a liquid bacterial culture grown for 24 h in nutrient broth or with 0.6 mL of a fungal spore suspension (1 × 10^6 spores/mL) were allowed to dry for 1 h. Test samples were then applied to 4 mm filter paper disks in 10 μL of MeOH. Disks were air-dried for 1 h and then placed on test plates. Plates were monitored for the development of zones of inhibition every 24 h for 4 days. Tetracycline (10 μg) was used as a positive control for the bacterial targets. Filipin (10 μg) was used as a positive control for the fungal targets.

Insecticidal Assay. Eggs of *Aedes aegypti* (Rockefeller strain) were hatched in tapwater under vacuum and then held at 25 °C for 24 h to supply second instar larvae for testing. Approximately 10 larvae were transferred into each well of a 24-well microtiter plate. Water transferred with larvae was then removed, and 1 mL of a test solution was added to the well. Test materials were added to 1 mL of water in 10 μL of MeOH; water alone and water with 10 μL of MeOH were used as the negative controls, and avermectin (10 μg) was used as the positive control. Larval mortality was monitored at 24 and 48 h.

***Salmonella* Mutagenicity Test.** The standard plate incorporation assay, as described by Maron and Ames (*19*), was used to test the mutagenic activity of **1** and **2**. *Salmonella typhimurium* TA100 was

Table 1. NMR Data for NG-391 (**1**) and NG-393 (**2**)

	1			2		
position	δ ^{13}C[a]	δ ^{1}H mult [J (Hz)][b]	HMBC[c]	δ ^{13}C[a]	δ ^{1}H mult [J (Hz)][b]	HMBC[c]
1	16.1	1.75 dd (7.3, 1.4)	2, 3	16.1	1.75 dd (7.2, 1.4)	2, 3
2	140.5	6.99 dq (7.2, 0.9)	1, 3, 4, 20	140.5	6.97 m	1, 3, 4, 20
3	130.5			130.2		
4	128.1	6.21 s	2, 5, 6, 20, 22	126.9	6.23 s	2, 5, 6, 20, 22
5	138.1			138.3		
6	142.4	6.59 d (14.9)	4, 5, 8, 22	142.3	6.55 d (15.1)	4, 5, 7, 8, 22
7	128.5	6.43 dd (15.2, 10.7)	5, 6, 8, 9	123.9	6.96 m	5, 6, 8, 9
8	143.6	6.77 dd (14.6, 10.9)	6, 7, 9, 10	139.2	6.52 t (11.1)	6, 7, 9, 10
9	127.9	6.64 dd (14.2, 11.1)	7, 8, 10, 11	124.0	6.39 t (11.2)	7, 8, 10, 11
10	145.6	7.47 d (11.3)	8, 9, 12, 23	138.5	7.88 d (11.8)	8, 9, 11, 12, 23
11	133.6			134.3		
12	190.2			189.6		
13	61.6			61.6		
14	63.8	4.03 d (2.5)	13, 15, 17	63.8	4.09 d (2.5)	13, 15, 17, 18
15	85.7			85.7		
17	170.0			169.6		
18a	35.9	2.09 m	14, 15, 19	35.8	2.10 ddd (14.6, 6.5, 4.0)	14, 15, 19
18b		2.11 m	14, 15, 19	35.8	2.17 ddd (14.6, 6.5, 4.0)	14, 15, 19
19a	58.4	3.96 m	15, 18	58.8	4.10 m	15, 18
19b		4.09 m	15, 18	58.8	3.98 ddd (10.8, 6.5, 4.0)	15, 18
20	167.6			167.7		
21	52.0	3.74 s	20	52.0	3.74 s	20
22	14.0	1.71 d (1.0)	4, 5, 6	14.4	1.70 d (1.2)	4, 5, 6
23	11.5	1.97 d (0.7)	9,[d] 10, 11, 12	11.2	1.99 d (1.0)	9,[d] 10, 11, 12

[a] From HMQC, HSQC, and HMBC spectra acquired in $CDCl_3$ with ^{1}H detection at 600 MHz. [b] Acquired in $CDCl_3$ at 600 MHz. [c] Numbers indicate carbons showing long-range correlations with the proton at the position designated in column 1. [d] Four-bond correlation.

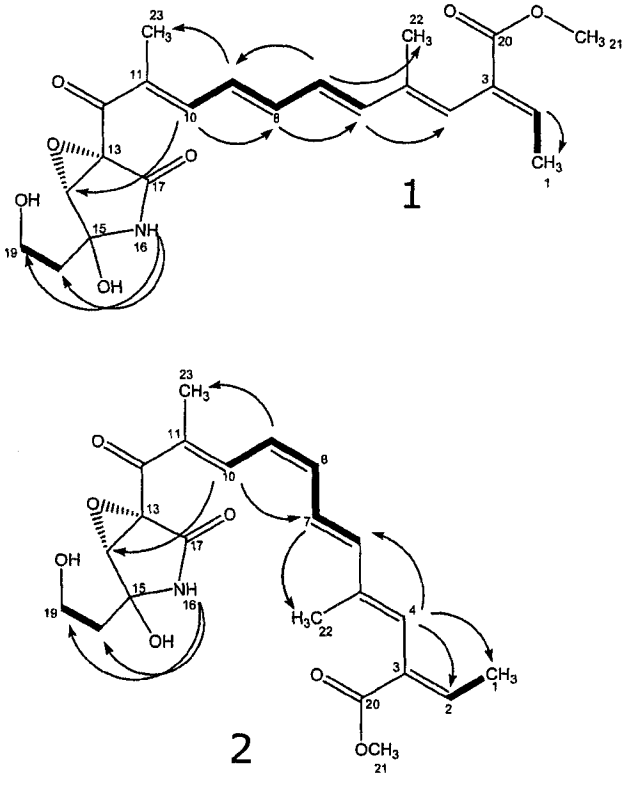

Figure 2. NMR data (600 MHz, $CDCl_3$) for NG-391 (**1**) and NG-393 (**2**). Key NOESY correlations are indicated by arrows. Bonds in boldface type indicate COSY networks.

used as the tester strain based on responses obtained with fusarins (*20*). Nutrient Broth and Bacto-Agar were obtained from BD-Difco, Inc. *S. typhimurium* tester strain TA100 was obtained from Moltox, Inc. (Boone, NC), as were 50× Vogel-Bonner Salts, 0.5 mM histidine/biotin solution, S9 fraction from Aroclor 1254-induced rats, S9 fraction buffers, 2-aminoanthracene (2-AA), and methyl methanesulfonate (MMS). Test compounds and positive controls were dissolved in ethanol (Sigma-Aldrich Chemical Co.). The positive controls, 2-AA and MMS, were tested at 5.0 and 600 μg/plate, respectively.

RESULTS AND DISCUSSION

Chemical Characterization. The two primary yellow components of a complex mixture of yellow compounds from broth extracts of *M. anisopliae* were identified as NG-391 (**1**) and NG-393 (**2**) following detailed analysis of mass and NMR spectra. HRESIMS data were consistent with the molecular formula $C_{22}H_{27}NO_7$ for both **1** and **2**. One-dimensional ^{1}H NMR spectra acquired in $CDCl_3$ were consistent with published data for **1** and **2** (*16*, *21*). Complete proton and carbon assignments, which have not been reported previously for these compounds, were made for **1** and **2** on the basis of COSY, HMQC (or HSQC), and HMBC data (**Table 1**). In addition, complete proton and carbon assignments made for **1** and **2** in CD_3OD supported the structural determinations (see the Supporting Information).

A doublet detected at δ 7.23 (J = 2.6 Hz) in a ^{1}H NMR spectrum of **1** ($CDCl_3$, 22 °C; 18.4 mM) was assigned to the N16 amide proton on the basis of HMBC correlations with C13–C15 and C17, NOESY correlations with H14 and the H18 methylene protons, and a COSY correlation with H14, which also exhibited an ~2.5 Hz coupling constant in the ^{1}H spectrum. In a similar fashion, a proton resonating at δ 6.77 in a ^{1}H spectrum of **2** ($CDCl_3$, 22 °C; 10.6 mM) was assigned to the amide proton based on an analogous set of HMBC, NOESY, and COSY correlations.

NMR signals produced by the two hydroxyl protons were revealed by intense exchange peaks observed in a NOESY spectrum of **1** ($CDCl_3$, 27 °C; 18.4 mM) between protons at δ 5.43 and 1.91. The downfield peak was observed as a singlet integrating as a single proton in the one-dimensional spectrum. The upfield peak was obscured in one-dimensional spectra by methyl signals. Analogous exchange peaks were observed in a NOESY spectrum of **2** ($CDCl_3$, 22 °C; 10.6 mM) correlating

resonances at δ 5.16 and 1.83. The downfield signal was assigned to the hydroxyl proton associated with C15 on the basis of its clear singlet nature. The upfield peak was assigned to the hydroxyethyl proton by process of elimination.

The amide (NH16), H14, and hydroxyl protons, and C15OH and C19OH of **1** and **2** exhibited temperature-dependent decreases in chemical shifts. The $\Delta\delta/\Delta T$ values (parts per billion per degree Celsius) for NH16, H14, C15OH, and C19OH were −12.3, −0.6, −22.1, and −11.8, respectively, for **1** and −9.1, −1.0, −14.1, and −4.6, respectively, for **2**. Predictably, the protons that exhibited temperature-dependent chemical shifts also exhibited a concentration-dependent effect. The $\Delta c/\Delta T$ coefficients estimated for NH16, H14, and the C15 hydroxyl proton of **1** ($CDCl_3$, T = 30 °C) were 17.6, 0.5, and 13 ppb/mM, respectively. The concentration dependence of the upfield hydroxyl signal could not be accurately determined due to excessive overlap with the H1 and H22 methyl proton signals. Similar concentration-dependent effects on chemical shifts of amide and hydroxyl protons were reported for fusarins (*22*).

These effects complicated comparisons of proton chemical shift data with published spectra (*16*, *21*), especially because of overlap of the NH16 signal with the signals from the olefinic protons. A practical benefit of acquiring ^{1}H spectra over a range of temperatures for these compounds was the ability to manipulate the chemical shift of the amide proton signal so that it was well-resolved from all other signals. This greatly facilitated interpretation of two-dimensional data, especially HMBC spectra in which the amide proton provided strong correlations with C13 and C17, which were only weakly detected by H14.

The 8*Z* geometry of **2** was indicated by the $^3J_{H8-H9}$ of ca. 11 Hz (**Table 1**) and confirmed by a strong NOESY correlation between H7 and H10 (**Figure 2**). This signal was not present in the NOESY spectrum of **1**, which instead showed the expected cross-peak between H10 and H8 (**Figure 2**) and a $^3J_{H8-H9}$ of ca. 14 Hz (**Table 1**) consistent with the all-*E* geometry of the conjugated pentaene moiety. Models of **2** were constructed using X-ray crystallographic coordinate data for (8*Z*)-fusarin C (*22*) as a starting point and then replacing the methyl group attached to C7 with a proton and varying the C−H bond length through the range observed for the neighboring olefinic protons (0.88−1.04 Å). In these models, H10 is closer to H6 (2.52 Å) than to either H9 (2.87 Å), H8 (3.53 Å), or H7 (3.28−3.43 Å). However, while NOESY and ROESY spectra of **2** showed a strong cross-peak between H10 and H7 and only weak cross-peaks between H10 and H9, there was no cross-peak between H10 and H6. These observations indicate that, in the solution state, the torsion angle about the C7−C8 single bond (C6−C7−C8−C9) in **2** is closer to the planar condition (Φ = 180°) than that observed in the solid state in (8*Z*)-fusarin C (Φ = 51.3°) (*22*) in which steric hindrance may prevent rotation about the C7−C8 single bond that would position the C7 methyl group closer to the pyrrolidinone moiety.

The crystal structure of (8*Z*)-fusarin C indicates an intramolecular hydrogen bond between the NH16 amide proton and the ester carbonyl oxygen atom attached to C20 (*22*). It also establishes the distance in the structure between the amide proton and H4 as being 2.48 Å. The lack of a NOESY correlation between NH16 and H4 in spectra of **2** and other features of the NOESY and ROESY spectra noted above suggest that the desmethyl analogue does not form an intramolecular hydrogen bond between NH16 and the C20 carbonyl oxygen in solution. The large negative $\Delta\delta/\Delta T$ values observed in both **1** and **2** for the NH are consistent with associative (intermolecular) hydrogen bonding (*23*).

Table 2. Quantitative Estimates of **1** and **2** in Crude Broth and Mycelial Extracts from Cultures of *M. anisopliae NPS1* Knockout Mutant KOB1-3, WT ARSEF 2575, the *NPS1* Knockout Mutant (KO8-18-1), and the Ectopic Control Transformant (EctA-18)

	broth[a]		mycelium[a]	
strain	**1**	**2**	**1**	**2**
KOB1-3	2.15(0.37)a	1.93(0.11)a	0.42(0.04)a	0.29(0.01)a
2575 (WT)	0.20(0.03)b	0.20(0.04)b	0.06(0.01)b	0.03(0.01)b
KO8-18-1	0.15(0.04)b	0.15(0.04)b	0.04(0.01)b	0.02(0.01)b
EctA-18	0.22(0.06)b	0.23(0.07)b	0.05(0.01)b	0.03(0.01)b

[a] Estimates are expressed as micrograms per gram of mycelial dry weight (mean ± the standard error of the mean; n = 3). Means within a column not followed by the same letter are significantly different at $P < 0.05$ in a Tukey's HSD test.

In the original identification of NG-391 and NG-393 from *Fusarium* sp. (*16*), the configurations at the three stereogenic centers, C13−C15, were not specified but optical rotations were reported for **1** (39.3°) and **2** (32.0°) in MeOH. Hayashi et al. (*21*) deduced the absolute configuration of the epoxide carbons of NG-391 as 13*R*,14*R* from a model synthesis but did not specify the configuration at C15. Nonetheless, they stated that their synthetic product has the same absolute configuration as the natural product on the basis of agreement of their optical rotation measurement (42.7°) with the value reported for the natural product (*16*). Our rotation measurements for **1** (76.1°) and **2** (181.6°) did not agree with these prior published values (*16*, *21*). If the published rotation data are from enantiomerically pure samples, then our values are not consistent with mixtures of enantiomers, as the presence of L antipodes of D enantiomers would reduce the positive rotations, not increase them. Therefore, we consider it unlikely that **1** and **2** are mixtures of enantiomers.

Hayashi et al. (*21*) indicated that their synthetic NG-391 was afforded as a mixture of epimers (13*R*,14*R*,15*S* and 13*R*,14*R*,-15*R*). The only ^{1}H NMR signals that are likely to differ significantly between the two forms would be those from the protons neighboring C15, i.e., the H18 and H19 methylene protons and the H14 methine proton. As noted above, the signals for these protons in **1** and **2** agree well with published data. However, spectra also indicate the presence of traces of related compounds. We postulate that the natural products from *M. anisopliae* also exist as a mixture of C15 epimers and that differences in the proportions in the mixture, along with possible contributions from other related compounds that are copurified as trace contaminants, account for the differences between our rotation values and those reported previously. Thus, on the basis of the preponderance of NMR evidence, the structures of **1** and, by analogy, **2**, are represented as established previously (*21*) with the configurations specified at C13 and C14, but not at C15 (**Figures 1** and **2**).

HPLC Quantification. Fermentation broth extracts from mutant strain KOB1-3 yielded ca. 10 times more **1** and **2** per unit mass of dry mycelium than WT ARSEF 2575 and the other mutant strains that were tested (**Table 2**). Destruxin levels did not differ significantly among the strains that were tested (*15*). Results for the mycelial fractions were similar, showing a 7−10-fold overproduction of **1** and **2** with no significant difference in destruxin levels. All strains that were tested produced ca. 4−6 times more **1** and **2** in the broth than in the mycelium, indicating that the difference between the overproducing strain and the others is due to a difference in production and not secretion. The weakest signal observed among the mycelial samples had a S:N ratio of 30, 3-fold stronger than the LOQ.

Table 3. Mutagenicity Testing of NG-391 (**1**) and NG-393 (**2**) in the *Salmonella* Mutagenicity Test Using Tester Strain TA100, with and without the 10% S9 Fraction

	NG-391 (**1**)		NG-393 (**2**)	
μg/plate	no S9[a]	10% S9[a,b]	no S9[a]	10% S9[a,b]
0.00	94.6 (1.15)	119 (6.51)	101 (6.01)	99.3 (4.67)
0.36	95.1 (6.30)	275 (23.7)	101 (4.53)	205 (16.9)
0.63	90.6 (1.46)	397 (17.2)	100 (6.83)	297 (22.9)
1.25	88.8 (3.56)	517 (42.2)	95.7 (5.67)	380 (22.3)
2.50	96.8 (7.31)	663 (100)	93.9 (0.97)	529 (17.4)
5.00	97.0 (9.83)	859 (44.4)	98.8 (3.95)	668 (29.7)
positive control[c]	983 (11.6)	976 (7.82)	982 (3.19)	852 (19.5)

[a] Results are shown as the mean number (standard error of the mean; $n = 3$) of revertant (mutant) colonies per plate. [b] The test of mutagenicity for the linear portion of the dose–response curve was significant at $P < 0.0001$. [c] Positive controls were methyl methanesulfonate (MMS) (600 μg) without the S9 fraction and 2-aminoanthracene (2-AA) (5 μg) with the S9 fraction.

Antibiotic Activity. At 96 h, neither **1** nor **2** exhibited detectable antibacterial or antifungal activity at 100 μg/disk in disk diffusion assays in which positive controls tested at 10 μg/disk yielded large (>5 mm) kill zones at 48 h.

Insecticidal Activity. Compound **1** produced no mortality at 100 ppm after 48 h in an insecticidal assay against *A. aegypti* larvae in which a positive control, avermectin, produced 100% mortality in 24 h at 10 ppm.

Mutagenicity. Both **1** and **2** exhibited strong mutagenic activity in the Ames assay at 0.36−5.0 μg/plate in the presence of exogenous metabolic activation (10% S9 fraction) with significant dose−response increases ($P < 0.0001$) observed for the linear portion of the curves for **1** and **2** (**Table 3**). For **1**, a 2.3-fold increase in the number of revertant colonies per plate was observed at a concentration of 0.36 μg/plate, and a 7.2-fold increase was observed at a concentration of 5.0 μg/plate in the presence of the 10% S9 fraction (slope = 440; R^2 = 0.89). For **2**, a 2.1-fold increase in the number of revertant colonies per plate was observed at a concentration of 0.36 μg/plate, and a 6.7-fold increase was observed at a concentration of 5.0 μg/plate (slope = 380, $R^2 = 0.96$). No mutagenicity was detected for either **1** or **2** in the absence of the S9 fraction (**Table 3**). Reversion frequencies for both the positive and negative controls were within the range of expected results (*19*).

The only prior reports of **1** and **2** are from two undescribed strains of *Fusarium* (*16*, *17*). Thus, *M. anisopliae* represents a new source of these compounds and the first outside the genus *Fusarium*. Compounds **1** and **2** are 7-desmethyl analogues of fusarin C and (8*Z*)-fusarin C, respectively. The latter are mutagenic mycotoxins originally characterized from *Fusarium moniliforme* (*22*). Subsequently, fusarin C and related analogues were reported from various *Fusarium* species (*24*−*26*), as well as *Nectria coccinea* (*27*). Overproduction of fusarins by mutant strains has also been reported (*28*).

It is surprising that the structural similarity of NG-391 and NG-393 to the fusarins did not immediately trigger studies of their genotoxicity. Our confirmation that their mutagenicity rivals that of fusarin C and its 8*Z* isomer (*18*) raises the question of whether these compounds will be worth pursuing for biomedical applications (*16*, *29*). Additional research will be conducted to determine more accurately the mutagenic potency of **1** and **2** and, also, to determine the spectrum of mutations induced by these two compounds.

In a risk assessment study emphasizing toxic secondary metabolites, it was stated that fungal biocontrol agents, including *M. anisopliae*, "do not pose a health risk" (*4*). The production of potent mutagens by a strain of *M. anisopliae* represents a potential risk that was not known at the time that this study was conducted. The relevance of the production of mutagens by a single strain to the use of *M. anisopliae* as a biocontrol agent for insect pests is currently unknown. These compounds have yet to be documented from isolates of *M. anisopliae* other than ARSEF 2575 and its laboratory derivatives. Although **1** and **2** are readily detectable in culture filtrates and mycelium of ARSEF 2575 grown in liquid culture, they have not been detected in exhaustive methanolic extracts of spores (data not shown), which constitute the primary commercial product. We are currently mounting a screening effort to determine whether these compounds occur among commercial strains and field-collected isolates of *M. anisopliae*. If these compounds are present in commercial strains cultured in vitro, future research should include determining whether these toxins occur in mycosed insects and cadavers, as well as on food plants treated with the biocontrol products. In addition, the environmental stability of these compounds, their persistence in soil, and their fate in the food chain should be quantitatively evaluated, and an appropriate risk−benefit analysis should follow collection of such study data.

The lack of obvious antibiotic or insecticidal activity raises the question of how these compounds serve the producing organism. Compounds with antimicrobial activity can readily be rationalized as functioning in a context of interspecific competition. For an insect pathogen, insecticidal activity may be indicative of an adaptation to overcome host resistance mechanisms. The mutagenic activity of **1** and **2**, dependent as it is on activation by enzymes and cofactors from mammalian liver cells, offers little in the way of clues about a function that is ecologically relevant to a fungus. If knockout mutants deficient in production of these compounds become available, phenotypes exhibited by these strains may point the way to an understanding of their adaptive significance.

It is unlikely that production of mutagenic mycotoxins by wild-type *M. anisopliae* would have come to light if not for the serendipitous discovery of an overproducing mutant strain. This observation raises questions about what environmental cues regulate the expression of the multifarious secondary metabolites that can be elaborated by a single fungal species and points to the possibility that unknown chemistries may be similarly revealed in functional studies of other fungi. The discovery of mutagens in this manner heightens the urgency to find more efficient ways to fully characterize the metabolomes of fungi in general and, in particular, those used for biological control.

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Supporting Information Available: ^{1}H NMR spectra ($CDCl_3$) of **1** and **2** and ^{1}H and ^{13}C NMR assignments (CD_3OD) for **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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